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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,219,683**, on October 28, 1997, by **JOHN D. STEEVES, JASON K. DYER AND HAND S. KEIRSTEAD**, for "Immunological Composition and its Method of Use to Transiently Disrupt Mammalian Central Nervous System Myelin to Promote Neuronal Regeneration".

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Date

IMMUNOLOGICAL COMPOSITION AND ITS METHOD OF USE TO TRANSIENTLY DISRUPT MAMMALIAN CENTRAL NERVOUS SYSTEM MYELIN TO PROMOTE NEURONAL REGENERATION

FIELD OF THE INVENTION

This invention relates to promoting the growth and/or regeneration of neurological tissue within the central nervous system (CNS).

BACKGROUND

Approximately 1,100 new spinal cord injuries occur each year in Canada, with the number rising to over 10,000 per year in the United States. These numbers increase five-times for patients suffering brain damage involving inhibition to neural growth following traumatic brain injury. The number patients with chronic spinal cord injuries in North America is on the order of 300,000, which again increases five-times for the number of chronic patients suffering from brain damage involving inhibition to neural growth following traumatic brain injury. Mostly young and otherwise healthy become paraplegic or quadriplegic because of spinal cord injuries. There are an estimated 200,000 quadriplegics in the U.S. Given the amount of care required, it should not be difficult to envision how health care costs associated with caring for patients with CNS damage is well over $10 billion a year for North America.

The majority of spinal cord injuries result from damage to the surrounding vertebral column, from

fracture, dislocation, or both. Much of the damage to the spinal cord is due to secondary phenomena which occur a few hours following the injury. At this point the resultant damage may be reversible. Consequently, a critical factor for recoverable function is the time from injury to the institution of therapy. Complete axonal disruption from the immediate trauma or secondary phenomena precludes recovery. A number of interventional therapies, including opiate antagonists, thyrotropin-releasing hormone, local cord cooling, dextran infusion, adrenergic blockade, corticosteroids and hyperbaric oxygen have been utilized, but are of questionable clinical value.

In higher vertebrates, axons within the differentiated adult nervous system (CNS) possesses a limited capacity for repair after injury. Spinal cord injuries often result in the permanent loss of voluntary movement below the site of damage. Axotomized fibers, proximal to the cell body, initiate regenerative growth that is subsequently aborted within a short distance (1-2mm), and often followed by retrograde degeneration. Although central nervous system axons will not regrow in the environment of the adult spinal cord, peripheral nerve grafts into the CNS provide a favorable environment through which CNS axons will anatomically regenerate (May, et al., Cajal's Degeneration and Regeneration of the Nervous System, History of Neuroscience Series #5, Oxford Univ. Press, NY and Oxford, pp. 769). These findings indicate that adult CNS neurons retain intrinsic growth properties and, given favorable environmental conditions, are capable of successfully reactivating growth programs.

Myelin is composed of the compacted plasma membranes of Schwann cells and oligodendrocytes

and its composition resembles that of any other plasma membranes in containing lipids, proteins and water, but the relative proportions and dispositions of these components are unique to myelin. Myelin is produced by CNS oligodendrocytes and is characterized by the expression of myelin basic protein (MBP). MBP is only associated with myelin and is one of the first proteins expressed at the onset of myelination of CNS axonal fibers. The onset of myelination in the embryonic chick spinal cord at E13 coincides with the transition from a permissive to a restrictive period for the functional repair of transected spinal cord. The first appearance of chick oligodendrocytes on the tenth and eleventh embryonic day of development (E10-E11) precedes the initial formation of myelin by 2-3 embryonic days and is characterized by the expression of galactocerebroside (GalC), the major sphingolipid produced by Oligodendrocytes. GalC comprises approximately 15 percent of the total lipid in human myelin and is highly conserved across specifies. Antisera against GalC has been shown to demyelinate CNS tissue *in vitro* (Dorfman, et al., Brain Res. 177:105, 1979) and optic nerve in vivo (Sergott, et al., J. Neurol. Sci. 64:297, 1984.

Recently, myelin-associated proteins that inhibit the anatomical growth of axons *in vitro* (Caroni and Schwab, Neuron 1:85, 1988) as well as the regrowth of axotomized corticospinal fibers in vivo (Schnell and Schwab, Nature 343:269, 1990) have been identified in rat spinal cord. The neutralization of some of these myelin-associated proteins with specific antibodies facilitated the anatomical regrowth of some of these transected axons.

Several recent studies have provided evidence that the presence of CNS myelin can inhibit the

regenerative growth of some severed CNS axons (c.f. Schwab and Bartholdi, 1996). Potential inhibitory molecules associated with myelin have also been identified, including NI35/250 (Caroni and Schwab, 1988a, b; Schnell and Schwab, 1990) and myelin associated glycoprotein (MAG; McKerracher et al., 1994; Mukhopadhyay et al., 1994). The *in vivo* presentation of the IN-1 monoclonal antibody, which was developed against NI35/250, has been shown to facilitate the regeneration of corticospinal axons (Schnell and Schwab, 1990; Bregman et al., 1995).

Adult spinal cord can be demyelinated *in vivo* via drugs (e.g. ethidium bromide), however, these drugs have non-specific deleterious effects on other cell types in the central nervous system (e.g., astrocytes). In addition, myelin deficient strains of mice and rats are readily available, but are of limited experimental value due to a shortened life span (most do not survive beyond a couple of weeks after birth). The presence of inhibitory components located or embedded in myelin are inhibitory to the regeneration of axonal growth after injury, therefore, it is desirable to transiently remove myelin and its inhibitory components to promote the repair of injured adult spinal cord. Consequently, there is a need for improved methods of disrupting myelin *in vivo* which enhance regeneration of neurological tissue. The present invention provides methods which address this need.

SUMMARY OF THE INVENTION

The invention provides a composition and method of use for promoting regrowth, repair and/or regeneration of neurons in the CNS of a mammalian subject, such as a human, by contacting a

therapeutically effective amount of an immunological composition comprising one or more complement fixing antibodies, or binding fragments thereof, which binds an epitope on myelin and one or more complement proteins or fragment thereof.

One embodiment of the invention provides an immunological composition that promotes regeneration of neurological tissue in a mammalian CNS comprising an antibody or binding fragment thereof, in combination with one or more components of serum complement proteins or active fragment thereof. The antibody can be directed towards toward almost any surface antigen of myelin and must be complement fixing or have complement protein covalently bound thereto.

Another embodiment provides the method of using the composition to stimulate immunological disruption of myelin or demyelination of axons, comprising administrating a therapeutically effective amount of the composition to the site of damage resulting from acute damage to CNS neurons. The method can be used following immediate or chronic injury.

A further embodiment provides the method of using the composition to stimulate immunological disruption of myelin or demyelination of axons, comprising administrating a therapeutically effective amount of the composition to an area of the mammalian CNS damaged as a result of degenerative disease, such as Alzheimer's or Parkinsons disease.

Another embodiment provides the method of using the composition to generate an environment within the mammalian CNS that is relatively permissive to growth of transplanted cells.

In yet another embodiment, labeled antibodies are provided enabling the detection and monitoring of therapeutic treatment using the composition and method of this invention.

In another embodiment, the invention provides a kit useful for the removal of myelin from neurological tissue within the CNS and thereby promoting regeneration of the neurological tissue.

FIGURES

Figure 1: Experimental methods. **A)** Drawing of a dorsal view of the rat central nervous system, indicating the relative origins and course of the rubrospinal tract (RN) and lateral vestibular tract (LVe). Also illustrated (solid line) is the left-side thoracic hemisection lesion (~ T10, line), the immunological infusion site (~ T11, vertical hatching), and the site of the Fluorogold injection (~L1, diagonal hatching). **B)** composite photomicrograph of parasagittal sections through the lower thoracic and rostral lumbar spinal cord (T9- L1, rostral is up). Some Fluorogold diffusion can be clearly emanating from the injection site as an intense white "halo", however, this staining rapidly decreased with distance from the site of injection and none was ever visible rostral to T11, the immunological infusion site (i.e. no diffusion to or above the lesion at T10, thus no evidence for any "false" positive retrograde labeling of brainstem-spinal projections). **C)** photomicrograph of a transverse section of spinal cord at the level of T10 left side hemisection lesion, stained with cresyl violet. All lesions were assessed and always resulted in severing the funiculi through which

the rubrospinal and lateral vestibulospinal tracts traverse. The contralateral dorsal (dh) and ventral (vh) horns were always left undamaged; the central canal (cc) is labeled for orientation. **D** and **E**) Non-specific fluorescence associated with blood cells within the lesion and pump implantation sites indicating the degree of damage associated with the lesion and cannula implantation, respectively. Specific Fluorogold fluorescence labeling was never observed at the level of the cannula implantation or hemisection injury.

Figure 2: Electron photomicrographs of transverse sections through the dorsolateral funiculus after continuous intraspinal infusion of immunological reagents for 7 days. **A**) Within one spinal segment (<2mm) of the infusion site, large regions of naked, demyelinated axons were visible. Some axons were observed to be associated with monocyte cells (M, e.g. infiltrating macrophage), some of which also contained myelin ovoids (arrow) or myelin debris. **B)** On other grids, monocytes and invading polymorphonucleocytes (PMN) could also be seen in close association with demyelinated axons. Macrophages were identified on the basis of their high density endoplasmic reticulum (arrow-heads), and "finger-like" processes. Some monocytes have laid down basal lamina components such as collagen (Col), which distinguishes them from astrocytes. Multi-lobed nuclei are characteristic of PMNs and facilitate their identification. **C**) Example of myelin-disruption often observed from 4-8mm (1-2 spinal segments) from the immunological infusion site where the axons were still associated with myelin, however, the myelin lamellae were disrupted (delaminated). Some regions of coherence in the myelin wrapping did persist (arrows). **D**) Example of the appearance of axons within the dorsolateral funiculus after a control infusion of Guinea-pig complement alone. Some non-specific damage of myelin

sheathes occurred, especially within one spinal segment of the infusion site, however, the compact nature of the myelin remained intact. Original magnification x 4 000 (A, C, D), x10 000 (B).

Figure 3: Regeneration of rubrospinal neurons after left-side thoracic hemisection and subsequent immunological myelin suppression treatment. Panels **A** and **B** are photomicrographs of rubrospinal neurons from the same experimentally-treated animal (14 days infusion of serum complement with anti-GalC); A is from the uninjured Red nucleus and B is of the injured Red nucleus. Panels **C** and **D** are also from same control-treated animal (14 days infusion of serum complement only); where C is the uninjured Red nucleus and D is the injured Red nucleus. Fluorogold injection within the rostral lumbar cord 28 days after injury resulted in the retrograde labeling of uninjured rubrospinal neurons (**A** and **C**) as well as those rubrospinal neurons that had regenerated from the injured Red nucleus (**B** and **D**), please see results for further details.

E and **F**) Axotomized rubrospinal neurons were retrograde labeled at the time of injury with the frist label, RDA (solid arrow heads) and subsequently 28 days later with the second label, FG (open arrow heads). Double-labeled (RDA+FG) cells are indicated by an asterisk and represent those rubrospinal neurons that had regenerated after immunological myelin-suppression treatment (for further discussion of double-labeling techniques and data , see Hasan et al., 1993).

G) Drawing of a transverse section through the midbrain indicating the location of the magnocellular division of the Red nucleus (RN-MC) that predominantly projects to the caudal cord. Aq = aqueduct, OM = oculormotor nuclei, SN = substantia nigra. Scale bar = 100μm

Figure 4: Regeneration of lateral vestibulospinal neurons after left-side thoracic hemisection and

subsequent immunological myelin suppression treatment. Panels **A** and **B** are photomicrographs of lateral vestibulospinal neurons from the same experimentally-treated animal (14 days infusion of serum complement with anti-GalC); A is of the injured lateral vestibular nucleus and B is from the uninjured lateral vestibular nucleus and. Panels **C** and **D** are also from same control-treated animal (14 days infusion of serum complement only); where C is the injured lateral vestibulospinal nucleus and D is the uninjured lateral vestibulospinal nucleus. Fluorogold injection within the rostral lumbar cord 28 days after injury resulted in the retrograde labeling of uninjured lateral vestibulospinal neurons (**B** and **D**) as well as those lateral vestibulospinal neurons that had regenerated from the injured lateral vestibulospinal nucleus (**A** and **C**), please see results for further details. Panel **E** is a drawing of a transverse section through the midbrain indicating the location of the lateral vestibular nucleus (LVe), SpVe = spinal vestibular nucleus, MVe = medial vestibular nucleus, 4V = 4th ventricle, FN = facial nerve tract, 7 = 7th cranial (facial) nucleus, PFl = paraflocculus. Scale bar = 100μm

Figure 5: Relative quantitative assessment of regeneration of rubrospinal and lateral vestibulospinal neurons after thoracic injury and immunological treatment. Regeneration was assessed by counting FG-labeled cells in alternating tissue sections; those with both multipolar neuronal morphology and FG labeling, were deemed to be positive. Percentage regeneration was calculated by comparison of the injured nucleus with the contralateral (uninjured) nucleus within the same animal. For each animal the degree of lesion was assessed. Filled bars, experimental; open bars, pooled control groups.

Figure 6: Quantitative assessment of regeneration of descending brainstem-spinal axons after chronic lateral hemisection & delayed immunological treatment. Percentages of retrogradely labeled red

nucleus (red) and lateral vestibular (green) neurons vs. Contralateral uninjured, after control (PBS, Ab, GpC) treatment (open bars) or immunological disruption/demyelination (filled bars). Expressed as percentage labeled cells in the injured nucleus vs. Uninjured contralateral.

Figure 7: Perinuclear infusion of BDNF fails to stimulate the regeneration of chronically injured (6 month) rubrospinal neurons. Quantitative assessment of regeneration of descending rubrospinal axons after thoracic lateral hemisection & delayed immunological treatment. Percentages of retrogradely neurons vs. Contralateral uninjured, after control (PBS, Ab, GpC) treatment (open bars) or immunological disruption/demyelination (filled bars); with either infusion of BDNF or vehicle (PBS) to the vicinity of the red nucleus.

DETAILED DESCRIPTION OF THE INVENTION

The present invention arose out of the unexpected discovery that the combination of antibodies which bind an epitope on a myelin producing glial cell and complement could be used for disruption and demyelination of the myelin sheath, such that repair and regeneration of mammalian neurological tissue is enhanced.

The present invention provides a composition and method of use for promoting regeneration of neurological tissue in a mammalian subject, such as a human, with a nervous system dysfunction by contacting the neurological tissue with a therapeutically effective amount of a composition which

contains a complement fixing antibody which binds an epitope on myelin and complement. However, uses of the composition in the field of veterinary medicine is also an embodiment of the present invention.

The term "dysfunction" when used to describe the therapeutic use of the invention encompasses any type of trauma to the nervous system. Such trauma can arise from physical injury, as well as disease.

The method of the invention is based on the unexpected discovery that exposure of myelin sheath associated with a nervous tissue dysfunction to complement-fixing antibody specific for myelin producing glial cell and complement enhances regeneration of mammlian neurological tissue and related functions. The composition of this invention is valuable as a therapeutic agent in cases in which there is injury or disease of the mammalian nervous system and there is a need to facilitate neuronal plasticity and the regrowth of neural connections. The neurological tissue is exposed to the myelin disrupting composition, according to the invention, as soon as possible, following, for example, a human spinal cord injury, where therapy is directed to the CNS. In addition, such pathological diseases in which the composition and method of the invention might be useful in facilitating regeneration and recovery of neurological tissue in the CNS include Parkinson's disease and Alzheimer's disease.

A preferred target for the antibodies used according to the invention is the myelin sheath. An especially preferred myelin sheath epitope for binding antibodies according to the invention is galactocerebroside (GalC). GalC is the major sphingolipid produced by oligodendrocytes and

Schwann cells. GalC comprises approximately 15 per cent of the total lipid in human myelin and is highly conserved across species. Antibody to GalC is effective for the demyelination of neurological tissue. Since inhibitory components located or embedded in myelin are inhibitory to the regeneration of axonal growth, removal of myelin from the site of injury (*eg.* spinal cord), therefore, promotes the regeneration of axons. Surprisingly, it has been found that disruption and/or demyelination according to the invention is transient such that remyelination of the effected nervous tissue occurs shortly thereafter.

The complement system is the primary humoral mediator of antigen-antibody reactions. It consists of at least 20 chemical and immunologically distinct serum proteins capable of interacting with one another, with antibody, and with cell membranes. The composition of the invention includes a complement solution which may be a physically distinct solution from the antibody preparation. The complement solution can be homologous or heterologous with respect to the subject. The disruption and/or demyelination requires antibody to bind to an epitope on the myelin in order to activate the complement.

As is known, early components of both the classical pathway and the alternative pathway of complement activation act locally to activate C3, which is the pivotal component of complement, whose cleavage leads not only to the assembly of membrane attack complexes but also to the recruitment of various white blood cells. The larger fragment of C3 is called C3b, which binds covalently to the surface of a target cell where it acts as a protease to catalyze the subseuent steps in the complement cascade, and is also recognized by specific receptor proteins on macrophages and

neutrophils that enhance the ability of these cells to phagocytose the target cell. In paricular, membrane-imobilized C3b triggers a further cascade of reactions that leads to the assembly of membrane attack complexes from the late components.

The term "composition" as used herein comprises one or more serum complement proteins, or fragments thereof, in addition to one or more antibodies, or binding fragment thereof, directed towards a myelin surface antigen. The complement portion of the composition may be comprised of only one protein or only one fragment of a complement protein, such as C3b. Alternatively, a complement protein, or fragment thereof, could be covalently or noncovalently attached directly to the myelin surface-antigen binding antibody, such that binding of the antibody to the surface of the myelin will trigger the endogenous immune system attack. The composition may optionally include other chemicals or drugs such as growth factors.

The term "demyelination" refers to the removal or breakdown of myelin in neurological tissue. Preferably, the demyelination process of the present invention occurs in the CNS, most preferably in the spinal cord. Demyelinating antisera with complement-fixing activity preferably target oligodendrocytes, the cell type responsible for CNS myelinogenesis. Demyelination consists of the removal of the myelin sheath, for example, surrounding the neuron, or surrounding the neuronal projections (e.g., the axons).

The term "disruption" refers to delamination or disruption of the three-dimensional conformation of myelin.

The term "neurological tissue" refers to neurons and other cells typically situated in the region of the nervous system, such as the spinal axis of the CNS. Regeneration of neurological tissue includes the regrowth of neurons which results in the reformation of neuronal connections, both anatomically and functionally. When the composition of the invention is contacted with neurological tissue, this refers to myelin sheath produced by glial cells in the central nervous system.

As used herein, the term "therapeutically effective amount" refers to an amount of complement-fixing antibody and complement sufficient to effectively disrupt and/or demyelinate the CNS so that repair and regeneration of neurological tissue and neuronal connections is enhanced. Generally, the therapeutic composition is administered at a range from about 0.03 mg antibody to about 0.6 mg antibody in a 20% to 30% complement solution per kg body weight; preferably from about 0.05 mg antibody to about 0.4 mg antibody in 20% to 30% complement solution per kg body weight; most preferably from about 0.1 mg antibody to about 0.3 mg antibody in 20% to 30% complement solution per kg body weight. The exact ratio of antibody to complement may vary depending on the circumstances. However, since the amount of complement activated is directly proportional to the number of antibody molecules which bind, it is possible to administer relatively high concentrations to complement in excess of the relative concentration of antibody. In addition, the particular concentration of antibody administered will vary with the particular dysfunction, and its severity, as well as such factors as the age, sex, and medical history of the patient. Those of skill in the clinical arts will know of such factors and how to compensate the dose ranges of the composition accordingly.

The term "antibody" as used in this invention includes intact molecules containing an Fc region, which are capable of binding the epitopic determinant. The antibody of the invention must be able to specifically bind a surface antigen of myelin and activate the complement system. The preferred antibody of the invention specifically binds a myelin sheath epitope, such as galactocerebroside (GalC), O4, Myelin Oligodendrocyte Glycoprotein (MOG), Myelin Associated Glycoprotein (MAG), or fragments thereof. Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities (polyclonal solution), as well as distinct monoclonal antibody preparations can be utilized. Monoclonal antibodies specific for a given antigen can be prepared by methods well known to those skilled in the art (for example, Kohler, et al, Nature 256:495, 1975; Current Protocols in Molecular Biology, Ausubel, et al., ed., 1989). Monoclonal antibodies specific for GalC can be selected, for example, by screening for hybridoma culture supernatant which bind with GalC. The antibodies can be IgG or IgM.

A proportion of the antibodies in the composition can optionally be conjugated with a radionuclide, or other useful label such as a magnetic particle that will enable monitoring of the progress of therapeutic treatment using the composition. Such complexes can be detected by radioactive imaging techniques such as gamma scintigraphy, emission computed tomography and single photon emission computed tomography, or the state of myelination can be monitored using magnetic resonance imaging.

The term "complement" as used herein includes fractions which are purified as well as those which are enriched in the proteins which comprise the complement system. Such preparations should take

into account the relative lability of complement and provide sufficient combination of factors to allow complete activation of the complement cascade to allow demyelination to occur. Further, the therapeutic compositions used according to the invention can be derived from species different from that species being treated for the dysfunction due to the fact that the compositions are introduced directly to the neural tissue (e.g., intrathecally).

A composition according to the method of the invention can be administered to a subject parenterally by injection or by gradual infusion over time. For example, the composition can be administered intrathecally or injected directly into the spinal cord. Most preferably, when the nervous system dysfunction is a result of injury, injunction of the composition of the invention to the subject should be as close in time to the time of the injury as possible.

Preparations for parenteral administration are contained in a pharmaceutically acceptable carrier which should be compatible with both the components of the composition and the patient. Such carriers include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include propylene glycol, polyethylene glycol, metabolizable oils such as, olive oil, or squalane, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/acqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Preferred as a carrier vehicle is artificial cerebrospiral fluid.

The materials for use in the method of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise a GalC beinding antibody. Alternatively, the antibody and complement may be present in the same container. The constituents may be present in liquid or lyophilized form, as desired.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

The following illustrates the ultrastructural sequence of spinal cord demyelination and myelin disruption after immunological treatment. Perhaps more importantly, we observed that the local infusion of serum complement along with an IgG complement-fixing GalC antibody in the low thoracic (T11) adult rat spinal cord, after a hemisection lesion at T10, facilitated axonal regeneration by some brainstem-spinal cord neurons.

EXAMPLE I

Surgical Spinal Transection and Transient Immunological Myelin Disruption:

Ten to 12 week old adult female rats (Sprague-Dawley), approximately 200g in weight, were anaesthetized with Ketamine/Xylazine (60mg/kg, 7.5mg/kg respectively). After a limited laminectomy at T10, a left-side spinal cord hemisection lesion was made with micro-scissors and the extent of the lesion was then confirmed by passing a sharp scalpel through the lesion site (Fig. 1). Immediately after the lesion, an intraspinal cannula was implanted at T11 (n=22 total) and connected to an Alzet osmotic pump (14 day) to subsequently deliver a continuous intraspinal infusion (@ 0.5µl/hr) of serum complement (GIBCO BRL, #19195-015, 33% v/v) along with a complement-fixing IgG antibody to galactocerebroside (either our own polyclonal antibody or Chemicon Intl. Ltd., #AB142, 25% v/v). Cannulae were held in place by means of dental acrylic applied to the vertebral bone. Muscle layers were then sutured over the dental acrylic, and the superficial tissue and skin closed. The extent of the hemisection lesion was always confirmed histologically at the end of the 5-week treatment and recovery period.

All control animals received an identical hemisection lesion and were then intraspinally infused via an osmotic pump, for the same time period, with either vehicle alone (0.1 M phosphate buffered saline, PBS, n=5), antibody alone (25% v/v, n=2), or serum complement alone (33% v/v, n=6). All surgical procedures and subsequent animal care protocols were in accordance with Canadian and UBC Animal Care Committee guidelines.

Electron Microscopy:

Tissue for ultrastructural analysis was obtained from 10-12 week old adult female Sprague-Dawley rats sacrificed 7 days after infusion of serum complement along with a complement-fixing IgG antibody to GalC (see above for details) via an osmotic pump. Animals were lethally anaesthetised with Ketamine/Xylazine (120mg/kg, 15mg/kg respectively), then perfused intracardially with 200 ml of 0.1M PBS (pH 7.4) followed by 100 ml of 4% glutaraldyhyde in 0.1M PB, (pH 7.3) and subsequently postfixed overnight in the same fixative. The infusion site and surrounding cord was cut into 1mm transverse blocks and processed to preserve rostral-caudal sequence. Blocks were washed in 0.1M sodium cacodylate buffer (24 hours), post fixed in 2% OsO4, dehydrated through ascending alcohols and embedded in Spurrs' resin according to standard protocols. Tissue blocks from experimental and untreated-control animals were processed in parallel. Thin sections (1μm) were cut from each block, stained with alkaline Toluidine Blue and examined under a light microscope. For electron microscopic examination blocks were trimmed and sections cut at 80-100nm, mounted on copper grids, stained with uranyl acetate and lead citrate and viewed under a Ziess EM 10C electron microscope (at 80kV).

Retrograde Neuronal Labeling:

Single label Studies-

Twenty-eight days after the hemisection lesion and consequently 14 days after completion of the intraspinal infusion of the immunological reagents, each adult rat was anaesthetized with

Ketamine/Xylazine (60mg/kg, 7.5mg/kg respectively). Fluorogold (FG, 100-150nl total volume, 5% w/v in sterile dH_2O; Fluorochrome Inc. Englewood, CO, USA) was injected (50-75nl) bilaterally into the middle of the spinal tissue at the L1 level, approximately 1cm caudal to the lesion site (Fig. 1).

Double Label Studies-

At the time of lesion, the hemisection site was packed with gel-foam soaked with 12% (w/v in sterile dH_2O) rhodamine-conjugated dextran amine (RDA, 10,000MW FluoroRuby, Molecular Probes) for 30 minutes. The gel-foam was then removed and the remaining surgical procedures were completed (as outlined above). After 28 days survival, all animals were anaesthetized with Ketamine/Xylazine (60mg/kg, 7.5mg/kg respectively) and FG (100-150nl total volume, 5% w/v in sterile dH_2O) was injected (50-75nl) bilaterally into the spinal parenchyma at the L1 level of the cord (n=6).

Analysis of Regeneration:

Seven days following the injection of the FG tracer into the lumbar cord, animals were lethally anaesthetised with Ketamine/Xylazine (120mg/kg, 15mg/kg respectively) and then perfused intracardially with 200 ml of 0.1M PBS (pH 7.4) followed by 100 ml of 4% paraformaldehyde in 0.1M PBS, (pH 7.3). The brain and spinal cord were then removed and postfixed overnight in the same fixative. Subsequently, each brain and spinal cord was cleared of fixative and cryo-preserved by placing the tissue in a series of sucrose solutions (15% followed by 21%). Coronal or parasagital sections were cut at 25µm thickness on a cryostat. The brainstem and spinal cord tissue sections were

examined under a Zeiss Axioskop with a 100W mercury bulb (excitation/emission wavelength at: FG, 365/420nm; RDA, 546/590nm; fluorescein, 490/515nm)

The two brainstem-spinal nuclei used to assess the axonal regenerative abilities of experimentally treated animals were the Red Nucleus (RN) (origin is contralateral to the hemisection) and the Lateral Vestibular (LVe) Nucleus (origin is ipsilateral to the hemisection). Spinal-projecting axons from each RN cross to the opposite side of the midbrain and descend throughout the spinal cord within the contralateral dorsolateral funiculus. This contralateral spinal projection pathway is known to be a completely lateralized tract with the possible exception of 2-5 % of the axons which may project to the cord via an ipsilateral route (Waldron and Gwyn 1969; Brown, 1974; Huisman et al., 1981; Shieh et al., 1983). The LVe tract projects from the dorsolateral pontine hindbrain, maintaining an exclusive ipsilateral course throughout the brainstem and the ventrolateral white matter of the spinal cord (Zemlan et al., 1979; Shamboul, 1980).

Using a single-blind protocol, the number of retrograde labeled neurons within the Red Nucleus (RN) (contralateral to the hemisection) and the Lateral Vestibular (LVe) Nucleus (ipsilateral to the hemisection) were counted in every other tissue section (throughout these brainstem nuclei) to avoid counting the same neuron twice. Only those cells exhibiting a nucleus, a neuronal morphology (i.e. multi-polar in appearance) and specifically labeled with FG (i.e. not visible using other fluorescent filters; see above) extending into the proximal processes, were deemed to be positively labeled spinal-projecting neurons. The percentage of regenerating neurons for each brainstem-spinal projection was then determined in comparison to the number of labeled neurons within the contralateral (uninjured)

control nucleus within the same animal.

Extent of Spinal Cord Demyelination and Myelin Disruption after Immunological Treatment

Direct intraspinal infusion over 7 days (@ 0.5µl/hr) of 33% heterologous (guinea pig) serum complement along with polyclonal antibodies to GalC (25%) in PBS resulted in extensive demyelination up to 2mm away from the infusion cannula (total rostrocaudal distance of 4mm or approximately 1 spinal segment (Fig. 2A). This zone of demyelination was bounded on either side by a further 8mm or 2 segments of spinal cord characterized by disrupted myelin (i.e. myelin that is extensively de-laminated, having an unraveled appearance, Fig. 2B). As shown in previous studies (Keirstead et al., 1992, 1995), control infusions of heterologous serum complement alone, myelin-specific antibody alone, or PBS alone resulted in only limited non-specific damage immediately centered around the cannula site. There was no surrounding zone of demyelination or myelin disruption (Fig. 2C).

The immunological demyelination and disruption of myelin within the experimentally-treated adult rat spinal cord was an active process extending throughout the entire cross-sectional profile of the cord. Immunological myelin disruption commenced within 1 day and was associated with an invasion of macrophages or resident microglia and polymorphonuclear cells (e.g. leukocytes such neutrophils, eosinophils and basophils). Many macrophages/microglia contained myelin fragments and completed their phagocytic activity within 7 days (Fig. 2D). This pattern of demyelination and myelin disruption could be maintained for as long as the serum complement and myelin-specific antibody were infused.

Recent evidence suggests that after the immunological infusion is terminated remyelination begins within 2 weeks (Keirstead and Blakemore, 1997; Dyer, Bourque and Steeves unpublished observations) and the new myelin originates from differentiating oligodendrocyte progenitors, although invading Schwann cells and surviving "mature" oligodendrocytes may also contribute to remyelination.

Choice of Retrograde Tracer and Its Diffusion Distance from the Injection Site

In this study, the major anatomical evidence for axonal regeneration within the hemisected and immunologically myelin-suppressed spinal cord of adult rats depends on a comparison between the number of retrogradely-labeled neurons within a homologous pair of brainstem-spinal nuclei. For these comparisons to be valid, the candidate brainstem spinal nuclei must have highly unilateral projections that are confined to one side of the spinal cord at all levels. As summarized in Fig. 1A, a left thoracic hemisection severed the contralaterally-projecting magnocellular neurons of the right red nucleus (RN), but left the projections from the left RN undamaged (as they project through the intact right dorsolateral funiculus of the thoracic cord). Likewise, a left thoracic hemisection severed the ipsilateral projecting neurons of the left lateral vestibulospinal nucleus (LVe), but left the axons from the right LVe nucleus undamaged (as they also project through the intact right side of the thoracic cord via the ventrolateral white matter).

If a retrograde tracer (single label) is injected into the rostral lumbar cord (1 cm caudal to the injury site), it should be incorporated and transported back to the cell bodies of origin by both intact axons,

as well as regenerated projections. Consequently, it is essential that the retrograde tracer reliably and extensively label most, if not all, descending spinal projection neurons. An equally important parameter is the tracer must be injected in a controlled and reproducible manner at a distance sufficiently caudal to the spinal injury to prevent any direct diffusion of the tracer to the level of the hemisection injury. The retrograde label that best satisfied all these conditions was Fluorogold (Sahibzada, et al., 1987). Fluorescent dextran amines, such as RDA, require a recent axonal injury to facilitate axonal uptake (c.f. Heimer and Zaborszky, 1989), and were therefore better suited for use in the double label retrograde-tracing studies (see description below).

In all cases, the Fluorogold label (100-150nl) was injected bilaterally within the rostral lumbar cord (1 cm or 2-3 spinal segments caudal to the hemisection injury site, Fig. 1). We assessed the time course and degree of rostrocaudal diffusion of Fluorogold within the lumbar and thoracic spinal cord of normally myelinated (control) animals and experimentally treated rats (i.e. under demyelinated and myelin disrupted conditions). Random 25µm sections of experimental and control-treated spinal cords (extending from L2 to T8) were examined under a fluorescent microscope using the highest intensity setting of the 100W mercury lamp. Spinal tissue was examined for the extent of Fluorgold diffusion at varying survival intervals after injection, including: 12hr (n=6), 24hr (n=6), 3d (n=6), 5d (n=6) and 7d (n=22). The maximum rostral diffusion distance observed was 4-6 mm (or 1- 1.5 spinal segments) and occurred within a time span of 24h. The degree of Fluorogold diffusion within the lumbar cord did not change over the subsequent time points examined (Fig. 1).

Evidence for Braintem-spinal Axonal Regeneration by Retrograde Neuronal Labeling

In brief, 28 animals; 12 experimental (9 retrogradely single-labeled, 3 double-labeled) and 16 control (13 retrogradely single-labeled, 3 double-labeled) were subjected to a left-side lateral hemisection of the T10 spinal cord. Immediately after hemisection, an infusion cannula (connected to a 14d osmotic pump) was inserted directly into the spinal cord 4-5 mm (1 spinal segment) caudal to the injury site. The osmotic pump contained one of a number of 3 different control solutions or the experimental treatment (i.e. PBS vehicle alone, serum complement alone, anti-galactocerebroside antibody alone, or serum complement with anti-GalC antibodies, respectively). Animals were then allowed to recover for 28 days before the Fluorogold was injected into the rostral lumbar, 1cm (i.e. at least 2 spinal segments) caudal to the lesion site. After a further 7 days survival, each animal was killed and the brain and spinal cord were removed for examination and analysis (see above for criteria used to determine a labeled neuron).

The extent of the hemisection lesion was assessed in every animal. In all but one experimental and one control-treated animal, the left thoracic spinal cord was hemisected (Fig. 1). Most importantly, the regions of the rubrospinal tract (dorsolateral funiculus) and the lateral vestibulospinal tract (ventrolateral funiculus) were severed. The right side white matter tracts were always remained intact and undamaged and usually the gray matter of the uninjured side was also undamaged.

As discussed above, the 2 pairs of brainstem-spinal nuclei examined for evidence of retrograde labeling (after spinal cord hemisection and immunological myelin suppression) were the RN and the LVe. These brainstem-spinal nuclei were chosen for their unilateral projection patterns within the thoracic and lumbar cord, enabling comparisons to be made between the retrograde-labeling within

an injured nucleus and the uninjured contralateral homologue. Comparing "blind" counts of the number of labeled neurons within each RN (Fig. 3A-B), the data indicated that 31.8% ± 4.7% (n=8, range 10-50%) of the injured magnocellular RN neurons had regenerated a sufficient distance into the caudal lumbar cord to incorporate and retrogradely transport the Fluorogold (Fig. 5). In contrast, control treated animals, receiving either the PBS vehicle alone, GalC antibody alone, or serum complement alone did not exhibit a significant amount of RN labeling; 1.49% ± 0.23%, (Fig. 3C-D; Fig. 5, n=13, range 0-3). The labeling of some neurons within the injured right RN nucleus may represent the small number of RN that do not project to the opposite side of the midbrain and descend within the ipsilateral (uninjured) cord (Shieh et al., 1983). No retrograde-labeling of cells was observed within the parvocellular region of the RN.

Retrograde-labeling of regenerating LVe neurons was also observed, but only after experimental demyelination and disruption of spinal cord myelin (Fig. 4). In 8 experimental animals, the mean percentage of regenerating LVe labeling, in comparison to the uninjured contralateral control nucleus, was 41.8% ± 3.1% (n=8, range 33-49%). In control-treated animals (see above) the percent LVe labeling was 2.24% ± 0.55% (Fig. 5, n=13, range 0-6).

Double retrograde labeling of the injured and myelin-suppressed rubrospinal tract was also qualitatively assessed (Fig. 5E and F). Large numbers of RDA-positive (first label) magnocellular RN neurons were observed after direct labeling of the lesion site at the time of hemisection injury to the thoracic spinal cord. After intraspinal myelin-suppression and subsequent injection of Fluorogold caudal to the lesion site (see above for details) a small overlapping population of FG-positive neurons

was observed (i.e. some neurons were labeled with both RDA and FG). Cells labeled exclusively by the first or the second tracer were also present in every brainstem analysed.

Examinations for any functional or behavioral differences during the 5 week recovery period after experimental treatment indicated no notable differences in locomotor patterns between injured animals and uninjured control animals (i.e. all animals walked and all animals were comparable with respect to basic reflex functions). These occurred regardless of the treatment infused intraspinally after a hemisection injury (e.g. PBS alone, GalC antibody alone, serum complement alone, or serum complement plus GalC antibody). Thus, subtle differences were very difficult to observe or quantify and 'gross' motor patterns were essentially the same.

As compared with prior art using spinal transection (Keirstead et al., 1992, 1995), the present invention is demonstrated using a hemisection model so that each animal could serve as its own internal control (i.e. axonal regeneration from injured brainstem-spinal projections could be readily compared to the uninjured contralateral homologue). In addition, the present invention strove to minimize the degree of cyst cavity formation that often occurs with larger spinal lesions, as well as the amount of animal discomfort over the relatively long recovery periods required.

The present invention also illustrates that the demyelination produced by the intraspinal infusion of serum complement and a myelin-specific antibody (e.g. GalC) produced a rapid and active demyelination over 1-2 segments of the cord with myelin disruption extending a further 2 segments, either side of the infusion site. Resident microglia and/or invading macrophages were observed to

contain myelin debris. The immunological suppression of spinal cord myelin surrounding the thoracic hemisection facilitated significant axonal regeneration by 2 unilaterally projecting brainstem-spinal pathways, the rubrospinal and lateral vestibulospinal (RN and LVe, respectively) tracts. Control treated animals (hemisection injury plus local intraspinal infusion of PBS alone, GalC antibody alone, or serum complement alone) showed little or no retrograde labeling within the injured RN or LVe.

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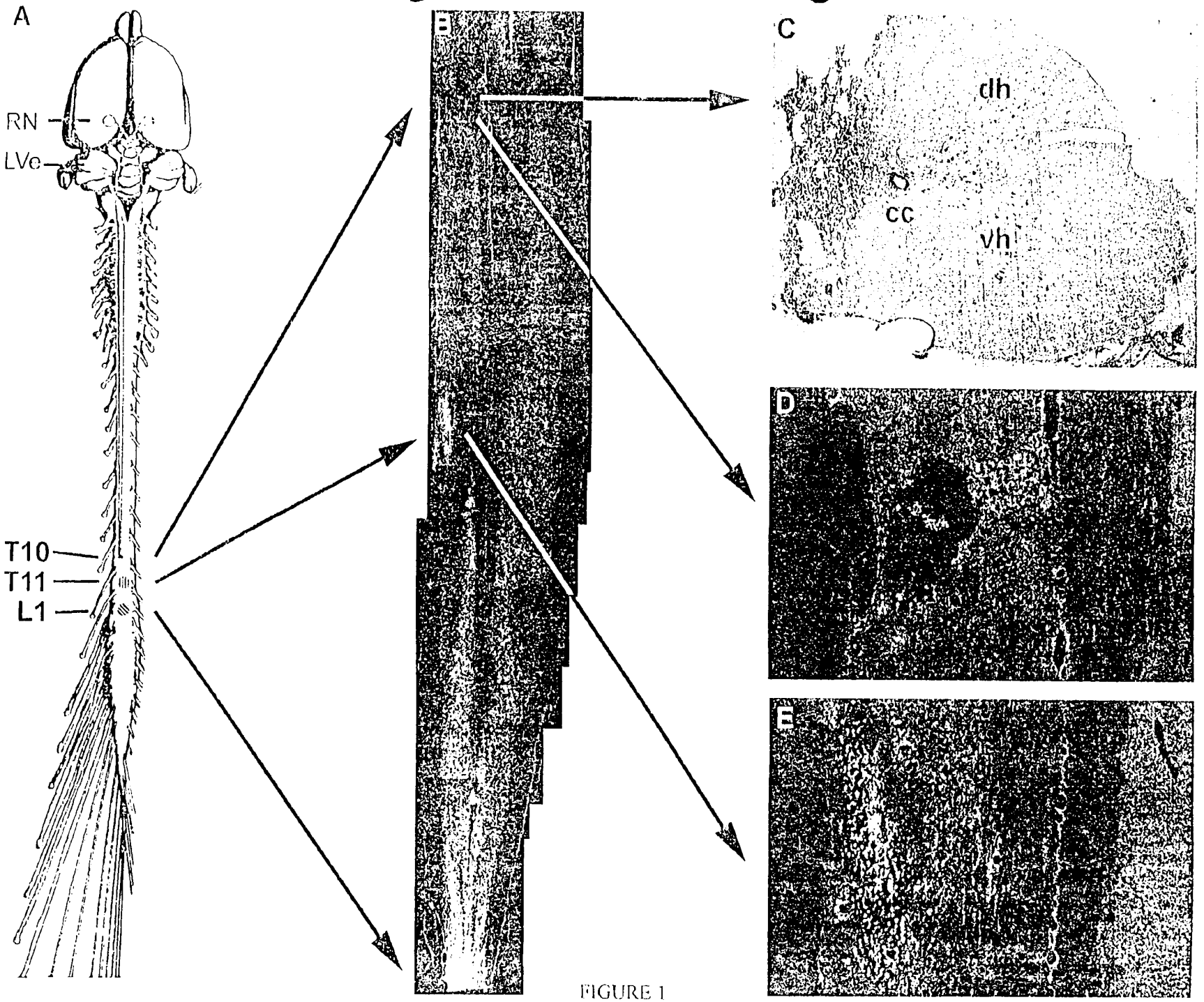

FIGURE 1

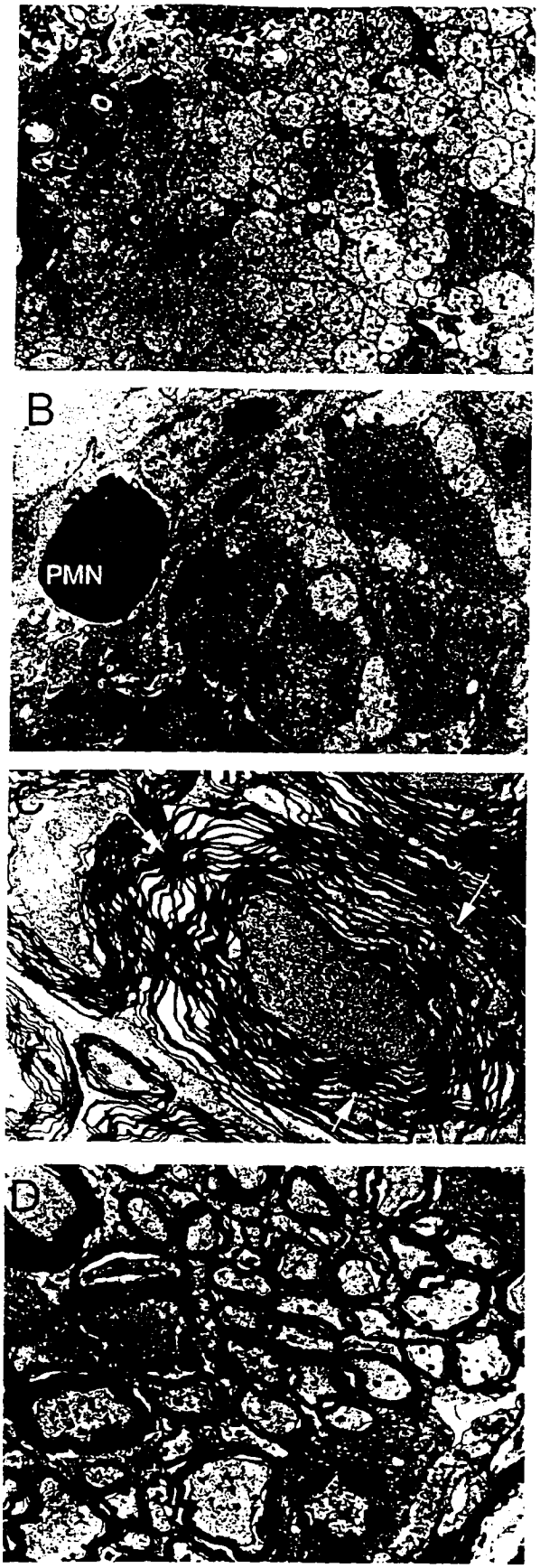

FIGURE 2

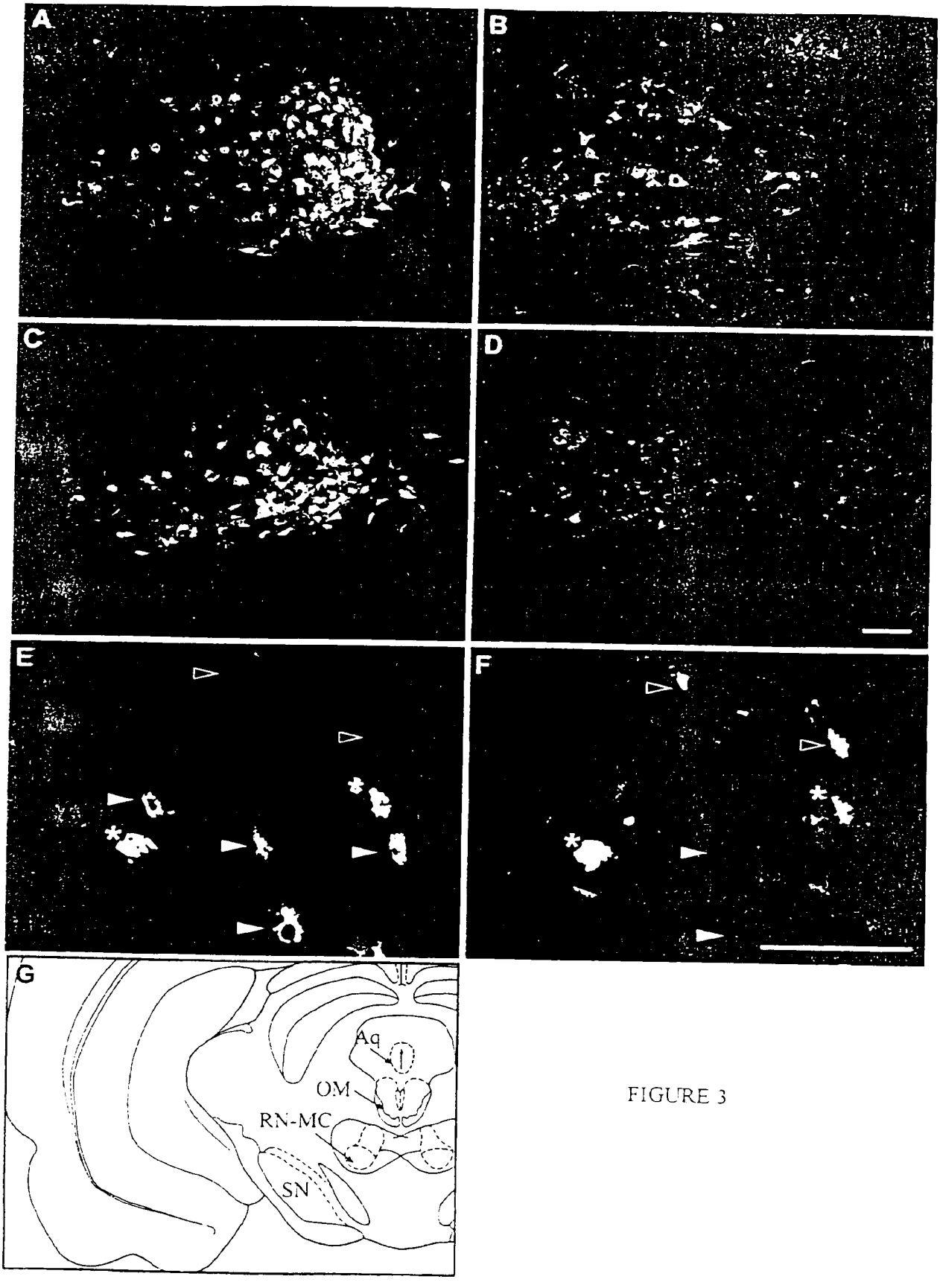

FIGURE 3

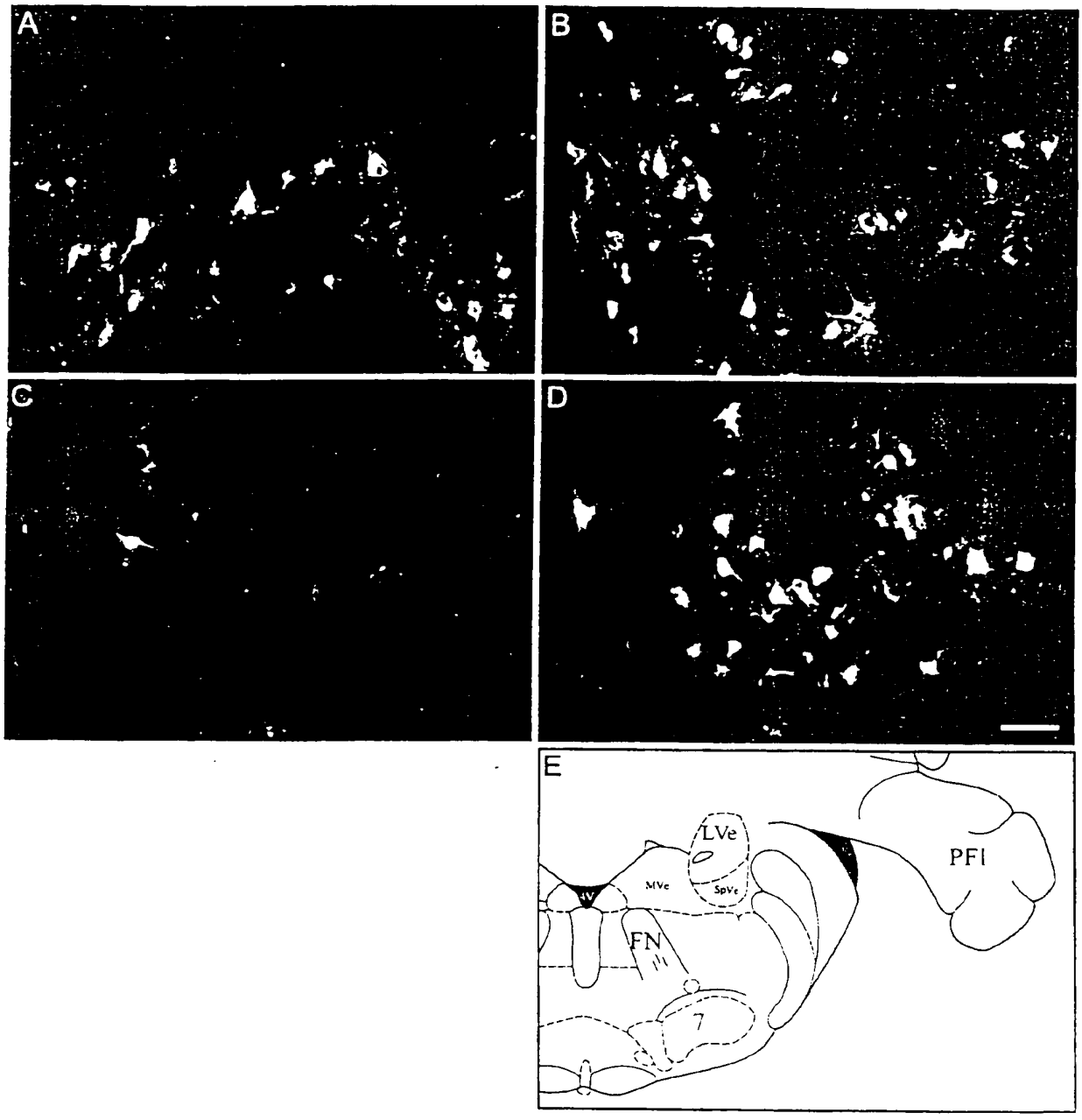

FIGURE 4

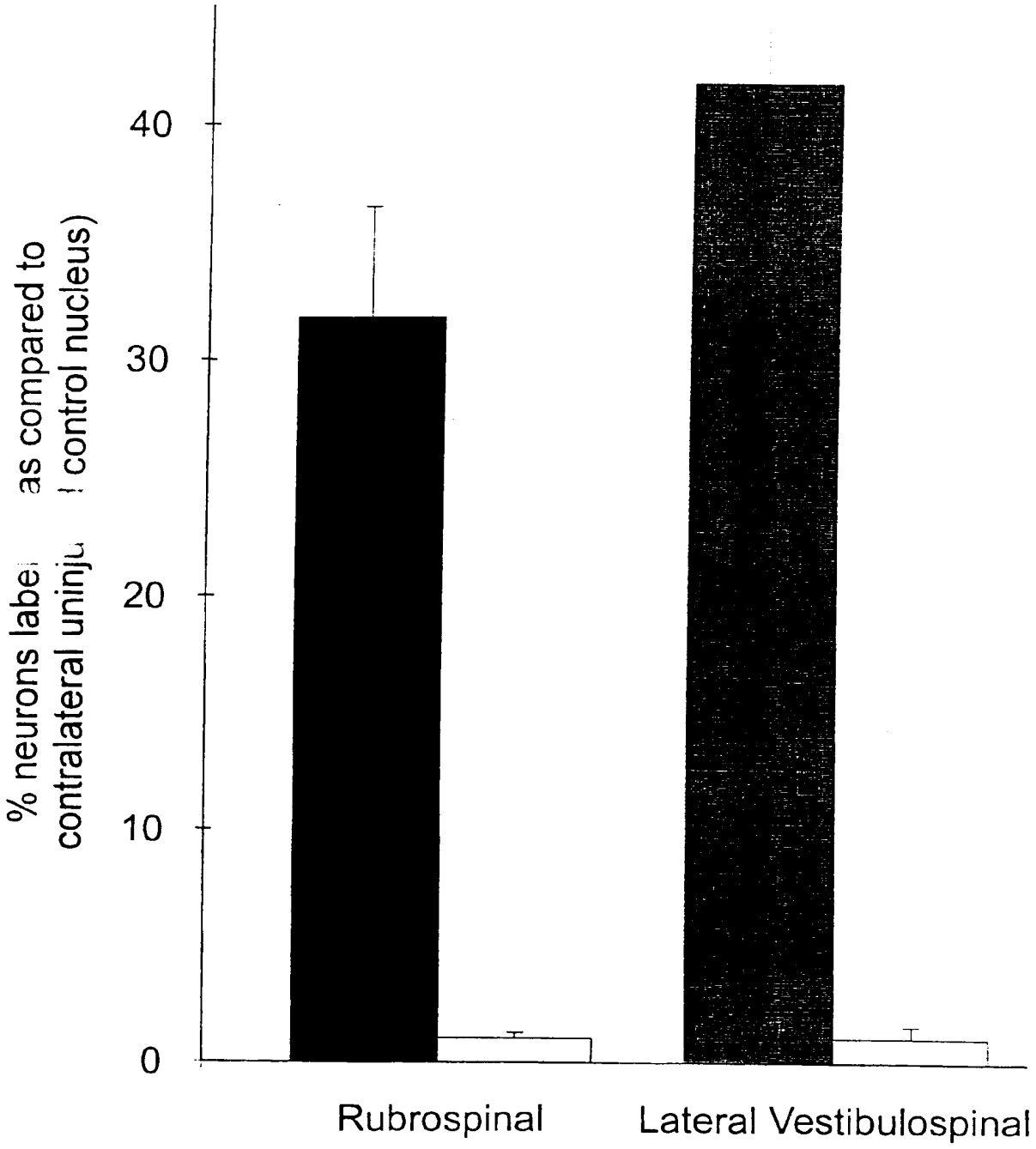
% neurons labe as compared to
contralateral uninju control nucleus)
40
30
20
10
0
Rubrospinal
Lateral Vestibulospinal

FIGURE 5

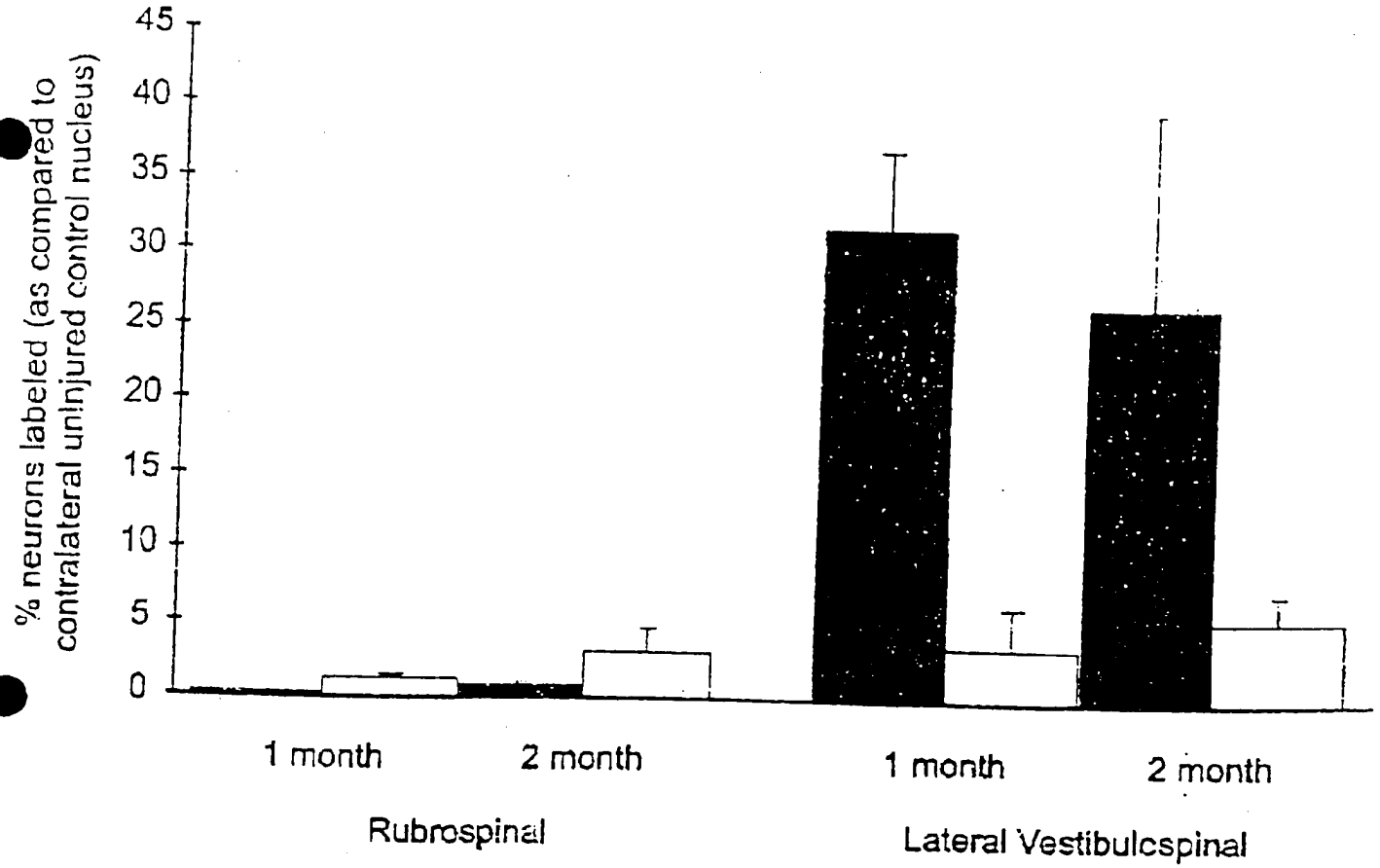
% neurons labeled (as compared to contralateral uninjured control nucleus)
45
40
35
30
25
20
15
10
5
0
1 month
2 month
1 month
2 month
Rubrospinal
Lateral Vestibulcspinal

FIGURE 6

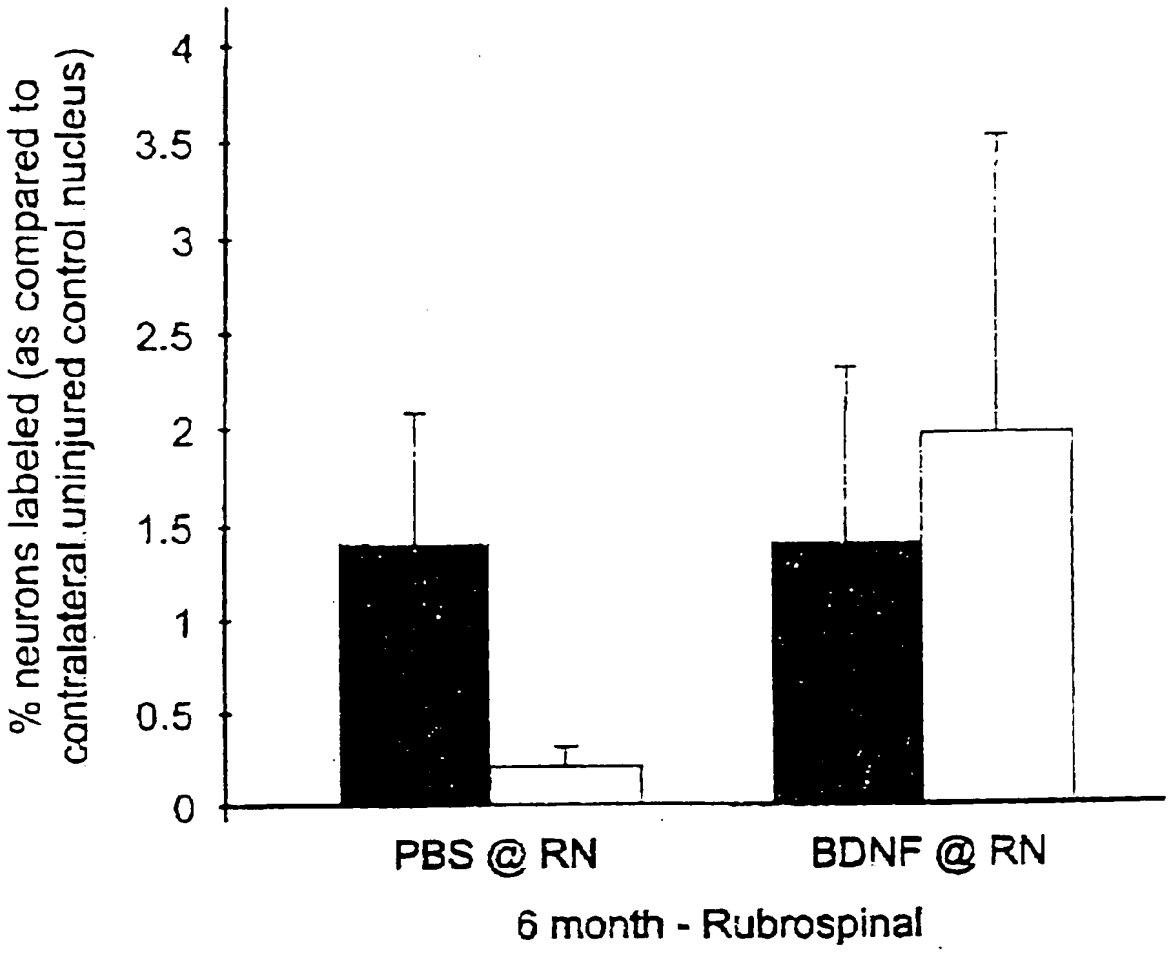
% neurons labeled (as compared to contralateral uninjured control nucleus)
4
3.5
3
2.5
2
1.5
1
0.5
0
PBS @ RN
BDNF @ RN
6 month - Rubrospinal

FIGURE 7